Identification of a Mutation in the Gene Encoding the α Subunit of the Stimulatory G Protein of Adenylyl Cyclase in McCune-Albright Syndrome

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Identification of a mutation in the gene encoding the α subunit of the stimulatory G protein of adenylyl cyclase in McCune–Albright syndrome

(*gsp* gene/oncogene/mosaicism/*café-au-lait* spot/endocrinopathy)

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ABSTRACT **McCune–Albright syndrome (MAS) is characterized by polyostotic fibrous dysplasia, *café-au-lait* lesions, and a variety of endocrine disorders, including precocious puberty, hyperthyroidism, hypercortisolism, growth hormone excess, and hyperprolactinemia. The diverse metabolic abnormalities seen in MAS share the involvement of cells that respond to extracellular signals through activation of the hormone-sensitive adenylyl cyclase system (EC 4.6.1.1). Mutations that lead to constitutive activation of $G_s\alpha$, the guanine nucleotide-binding regulatory protein that stimulates adenylyl cyclase activity, have been identified in a subset of human growth hormone-secreting pituitary tumors and human thyroid tumors. We report here the identification of a mutation in the gene encoding $G_s\alpha$ in a patient with MAS. Denaturing gradient gel electrophoresis was used to analyze amplified DNA fragments including exon 8 or exon 9 of the $G_s\alpha$ gene. In one subject with MAS a G-to-A transition was found in exon 8 of one of the two alleles encoding $G_s\alpha$. This single-base substitution results in the replacement of arginine by histidine at position 201 of the mature $G_s\alpha$ protein. Semiquantitative analysis of amplified DNA indicated that the mutant allele was less prevalent than the wild-type allele in peripheral leukocytes and was present in very low levels in skin. These findings support the previous contention that the segmental distribution and variable expression of the cutaneous, skeletal, and endocrine manifestations of MAS reflect an underlying somatic mosaicism. Further, these results suggest that the molecular basis of MAS is a postzygotic mutation in $G_s\alpha$ that causes constitutive activation of adenylyl cyclase.**

McCune–Albright syndrome (MAS) is characterized by the clinical triad of cutaneous hyperpigmentation, polyostotic fibrous dysplasia, and endocrine dysfunction. The pigmented cutaneous lesions are *café-au-lait* spots with irregular ("coast-of-Maine") outlines. The lesions typically display a segmental distribution that frequently follows the lines of Blaschko (1). Happle has suggested that this distribution of the cutaneous lesions reflects an underlying mosaicism and has proposed that MAS results from a postzygotic somatic cell mutation (1, 2).

An analogous pattern of variable involvement of hormonally responsive cells occurs in subjects with MAS. The metabolic abnormalities are characterized by excessive function of the responsive cells, and they are associated with endocrine syndromes, including precocious puberty, hyperthyroidism, hypercortisolism, growth hormone excess, and hyperprolactinemia. The peculiar bone lesion of MAS, polyostotic fibrous dysplasia, bears considerable resemblance to the skeletal changes that occur in primary hyperparathyroidism. These diverse metabolic abnormalities share the involvement of cells that respond to extracellular signals through activation of the hormone-sensitive adenylyl cyclase system (EC 4.6.1.1), the membrane-bound enzyme that catalyzes the formation of the intracellular second messenger cyclic AMP. However, these metabolic disturbances are not accompanied by elevated plasma concentrations of the relevant trophic or stimulatory hormones. Thus, MAS patients with precocious puberty demonstrate ovarian enlargement and follicular hyperplasia but have low serum levels of luteinizing hormone and follicle-stimulating hormone and a prepubertal response of luteinizing hormone to luteinizing hormone-releasing hormone (3). Patients with hyperthyroidism have thyroid hyperplasia but have suppressed levels of thyrotropin (4). The few patients with cortisol excess have adrenal hyperplasia and undetectable levels of adrenocorticotropin (5). Finally, excessive secretion of growth hormone (GH) by subjects with MAS is indistinguishable biochemically from that which occurs in patients who have autonomous GH-secreting pituitary tumors (6). These observations have led to the speculation that MAS is caused by a lesion that results in constitutive activation of adenylyl cyclase.

Activity of adenylyl cyclase is regulated by at least two guanine nucleotide-binding (G) proteins; G_s is responsible for stimulation of catalytic activity, whereas another group of G subunits, represented by at least three forms of G_i (7), mediate inhibition of the enzyme (8). Recent studies have demonstrated that mutations in the gene encoding the α subunit of G_s ($G_s\alpha$) can cause human disease. Inherited defects in the $G_s\alpha$ gene have been identified in patients with Albright hereditary osteodystrophy (9, 10). These mutations cause deficient activity of the $G_s\alpha$ protein and are associated with decreased responsiveness of diverse tissues to hormones that act through stimulation of adenylyl cyclase (11). By contrast, activating mutations in the $G_s\alpha$ gene have been identified in a subset of human GH-secreting pituitary tumors (12) and autonomously functioning human thyroid tumors (13, 14). These somatic mutations cause amino acid substitutions for Arg^{201} in exon 8 and Gln^{227} in exon 9. These abnormal $G_s\alpha$ proteins have markedly reduced GTPase activity and are able to stimulate adenylyl cyclase constitutively. These observations led to the concept that activating mutations of the $G_s\alpha$ gene convert it into a putative oncogene termed *gsp* (12).

In the present study we sought to identify presumptive activating mutations in the gene encoding $G_s\alpha$ in a patient with MAS. We used the polymerase chain reaction (PCR) to amplify exons 8 and 9 of the $G_s\alpha$ gene, and we analyzed the

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Abbreviations: MAS, McCune–Albright syndrome; $G_s\alpha$, α subunit of the stimulatory guanine nucleotide-binding regulatory protein of adenylyl cyclase; U, unit(s); DGGE, denaturing gradient gel electrophoresis; SNuPE, single-nucleotide primer extension; GH, growth hormone.

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DNA fragments by denaturing gradient gel electrophoresis (DGGE). To ascertain the presence of mosaicism, we examined DNA prepared from skin biopsy samples of a *café-au-lait* lesion and an area of unaffected skin as well as DNA from the patient's peripheral blood leukocytes.

METHODS

Subject. M.G. is a highly articulate, intelligent, 53-year-old White man with a history of recurrent pathological fractures and hyperthyroidism treated with ^{131}I ablation at age 35. Clinical examination revealed multiple bony deformities and short stature (122 cm). Multiple large *café-au-lait* lesions with irregular borders were present, predominantly over the left thorax. The skull was markedly misshapen with prominent supraorbital ridges and mandibular and maxillary asymmetry. The thorax was asymmetric and all four limbs were markedly deformed. Radiographs showed multiple cystlike expansile lesions with osteosclerosis. Shepherd's crook deformities were present in both femora. Histologic examination of bone showed fibrous dysplasia. Laboratory studies were notable for total serum calcium 8.8 mg/dl, serum phosphate 1.9 mg/dl, serum alkaline phosphatase 267 international units (U)/liter, serum osteocalcin 62 ng/ml (4.5–10.5 ng/ml), and intact parathyroid hormone 17 pg/ml (10–65 pg/ml), in which the numbers in parentheses are the normal range. Serum somatomedin C was 0.47 U/ml (0.34–1.9 U/ml) and total serum testosterone was 629 ng/dl (275–875 ng/dl). Serum thyroxine was 5.0 μg/dl, triiodothyronine resin uptake was 33.2%, and thyrotropin was 0.29 μU/ml. Basal urinary cAMP was elevated at 11.7 nmol/dl of glomerular filtrate [1.2–3.6 nmol/dl (15)] and the maximal tubular phosphate reabsorption (normalized to glomerular filtration rate) was markedly depressed at 1.1 mg/dl (2.8–4.5 mg/dl).

Amplification of DNA and cDNA by PCR. Genomic DNA was isolated from leukocytes as previously described (16); two separate blood samples were collected from the patient M.G. and processed in parallel. Genomic DNA was isolated by shave biopsies of a *café-au-lait* lesion and an area of normal skin by digesting the skin samples with collagenase (Sigma), 0.5 mg/ml in 10 mM Tris·HCl, pH 7.4/1 mM EDTA (TE), for 2 hr at 37°C and then with proteinase K (Sigma), 0.5 mg/ml in TE with 1% SDS and dithiothreitol at 10 mg/ml, for 16 hr at 37°C. The samples were then heated to 95°C for 10 min and extracted three times with 1:1 mixture of phenol and chloroform/isoamyl alcohol (24:1). The DNA was collected and washed once in a Centricon 100 microconcentrator (Amicon). RNA was isolated from skin biopsy samples by the guanidine isothiocyanate method and used as a template for first-strand cDNA synthesis in a 20-μl reaction volume containing 100 pmol of random hexamer primers and 200 U of reverse transcriptase from Moloney murine leukemia virus (BRL) (17). Oligonucleotide primers with sequence identity to the introns flanking exons 8 and 9 of *GNAS1* (Table 1) were synthesized on a Milligen/Biosearch Cyclone Plus DNA synthesizer. One member of each pair of primers had a 40-base 5′ extension of guanosine and cytidine residues (GC-clamp) for improved resolution on denaturing gradient gel electrophoresis (18). PCR was performed in a 100-μl volume with either 1 μg of genomic DNA or 1/10 vol of the synthesized cDNA as template. Reaction mixtures contained 50 pmol of each primer, 2.5 U of AmpliTaq DNA polymerase (Perkin–Elmer/Cetus), 100 μM each dNTP, 1.5 mM $MgCl_2$, 50 mM KCl, 10 mM Tris·HCl at pH 8.3, and gelatin at 10 μg/ml. A programmable thermal cycler (MJ Research, Watertown, MA; PTC-100) was used to perform 40 cycles of denaturation for 1 min at 94°C, annealing for 1 min at 57°C for exon 8 or 62°C for exon 9, and extension for 2 min at 72°C. The total final extension time was 7 min at 72°C.

Electrophoresis of Amplified Products. PCR fragments amplified from genomic DNA were analyzed by electrophoresis through 7% polyacrylamide gels and visualized with UV light after staining with ethidium bromide (see Fig. 1*A*). Amplified exons were analyzed by DGGE according to the method of Myers *et al.* (19) using 6.5% polyacrylamide gels (37.5:1 acrylamide/bisacrylamide) in TAE (40 mM Tris/20 mM sodium acetate/1 mM EDTA, pH 7.4) containing a linear gradient of urea and formamide. Optimal resolution of the PCR fragments was obtained on gels with denaturing concentration gradients of 30–60% for exon 8 or 50–80% for exon 9 (Fig. 1*B*), in which 100% denaturant was 40% (vol/vol) formamide/7 M urea.

DNA Sequence Analysis. Exon 8 fragments amplified from DNA from the patient's leukocytes were subcloned in the *Hph* I site of pCR1000 (Invitrogen). Plasmid DNA was prepared from isolated colonies by the alkaline lysis miniprep method (ref. 20, pp. 368–369). To distinguish normal and mutant alleles, primers AM-17 and MAL-63 were used to amplify the subcloned exon 8 fragments and the amplified fragments were analyzed by DGGE. Double-stranded plasmid DNA was sequenced by the dideoxy chain-termination method (Sequenase Version 2.0; United States Biochemical) using the m13 reverse primer (see Fig. 2).

Restriction Endonuclease Analysis. Exon 8 was amplified in a PCR reaction in which one primer (AM-17) had been 5′ end labeled with [^{32}P]dATP by using T4 polynucleotide kinase (BRL) (ref. 20, p. 122). Amplified fragments were digested with *Nla* III (New England Biolabs) and electrophoresed through 12% polyacrylamide gels. The restriction fragments were visualized by autoradiography (see Fig. 3).

Allele-Specific PCR. A primer (WFS-2) was synthesized with a 3′ terminus that was complementary to the G-to-A transition in the codon for Arg^{201} (Table 1). WFS-2 also contained a second internal mismatch that was required for specificity. PCR was performed as described above, with 250–500 ng of genomic DNA at an annealing temperature, 64°C, that allowed specific amplification of the mutant allele from genomic DNA (see Fig. 4*A*).

Single-Nucleotide Primer Extension (SNuPE). PCR fragments spanning exon 8 were amplified from genomic DNA with AM-17 and MAL-63 and were purified by electroelution from 7% polyacrylamide gels. A primer (WFS-6) was synthesized with its 3′ terminus adjacent to the identified mutation (Table 1). The fragments were combined with WFS-6, 10 μCi of [^{32}P]dTTP (3000 Ci/mmol, Amersham; 1 Ci = 37 GBq) and 2.5 U of AmpliTaq in 100 μl of the PCR buffer described above. The SNuPE was carried out by denaturing at 94°C for

Table 1. Primers used in this study

Primer	Sequence (5′→3′)	Description
AM-17	CTCTGAGCCCTCTTTCCAAACTAC	5′ exon 8
MAL-63	$(GC)_{40}$GGTTATTCCAGAGGGACTGGGGTGAA	3′ exon 8 with GC-clamp
MAL-32	$(GC)_{40}$GACATTCACCCCAGTCCCTCTGGAAT	5′ exon 9 with GC-clamp
AM-11	AAGCGTTCTTTACGAACAGCCAAGC	3′ exon 9
WFS-2	GATTCCAGAAGTCAGGA**G**AT	3′ allele-specific primer (long)
WFS-5	CAGAAGTCAGGACAT	3′ allele-specific primer (short)
WFS-6	GATTCCAGAAGTCAGGACA	5′ SNuPE primer

Boldface indicates a mismatch, as discussed in the text.

1 min, annealing at 57°C for 1 min, and extending at 72°C for 7 min. The primer extension products were electrophoresed through 12% polyacrylamide/7 M urea denaturing gels (21) and visualized by autoradiography (Fig. 4*B*).

RESULTS

Leukocyte genomic DNA from patient M.G., two normal subjects, and an additional subject with MAS was amplified with primers AM-17 and MAL-63. This produced a 295-base-pair (bp) fragment of the $G_s\alpha$ gene that contained exon 8, portions of the flanking intron sequences, and a 40-bp GC-clamp at the 3′ end (Fig. 1*A*). As shown in Fig. 1*B*, DGGE of the exon 8 fragments from normal subjects (lanes 7 and 8) demonstrated single bands. By contrast, DGGE of the exon 8 fragment amplified from M.G.'s leukocyte DNA demonstrated four bands (lanes 4 and 5), indicating that M.G. was heterozygous for a DNA alteration at this locus. The lowest band comigrated with the amplified DNA from normal subjects and represented a homoduplex derived from the normal allele. The next lowest band represented a homoduplex derived from the altered allele; the two highest bands represented heteroduplexes of the wild-type and variant alleles. DGGE analysis of an exon 8 fragment amplified from leukocyte DNA of a second patient with MAS revealed a single band with normal mobility (lane 6), suggesting that an exon 8 base alteration is not present in the leukocytes of all patients with MAS.

Amplification of genomic DNA with primers MAL-32 and AM-11 produced a 189-bp fragment that included exon 9 of the $G_s\alpha$ gene, flanking intron sequences, and a 40-bp GC-clamp at the 5′ end (Fig. 1*A*). DGGE analysis of the exon 9 fragments revealed a single band in both subjects with MAS and all normal individuals (Fig. 1*B*).

DNA sequence analysis of two independent subclones containing the abnormal exon 8 fragments from M.G. revealed the presence of a single G-to-A transition within the codon for Arg^{201}. This mutation changes the normal CGT to CAT and results in the substitution of histidine for arginine ($Arg^{201} \rightarrow$ His). DNA sequence analysis of the normal exon 8 fragment

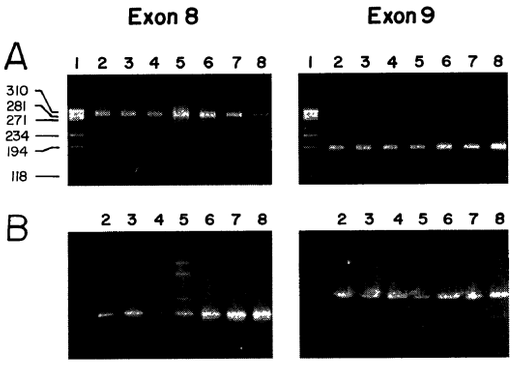

FIG. 1. Electrophoresis of PCR fragments. (*A*) Polyacrylamide gel electrophoresis of PCR products encompassing exon 8 or exon 9, amplified from a *café-au-lait* lesion (lane 2), normal skin (lane 3), or peripheral leukocytes (lanes 4 and 5) of the patient M.G., and from peripheral leukocytes of a second patient with MAS (lane 6) and two normal subjects (lanes 7 and 8). Note all fragments migrate as a single band. Lane 1 contains markers, whose lengths are indicated in bp. (*B*) DGGE of the same fragments. Note exon 8 fragments amplified from the patient's leukocytes migrate as four bands, two heteroduplex bands (upper) and two homoduplex bands (lower), indicating the presence of two different alleles. Note also that the intensity of the wild-type allele (lowest band) is greater than that of the mutant allele (next lowest band) in DNA amplified from the patient's leukocytes and that no mutant or heteroduplex bands can be detected in the patient's skin by this method. A single homoduplex band is present after DGGE analysis of exon 9 fragments from all DNA samples.

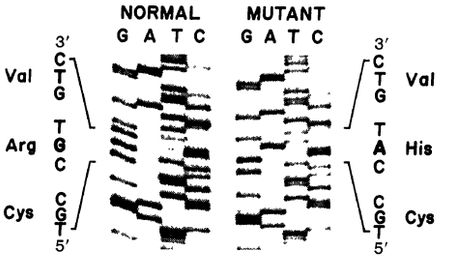

FIG. 2. DNA sequence analysis. Note the G-to-A transition in the codon for arginine at position 201. The mutant allele encodes histidine at this position. Note also the presence of an *Nla* III restriction endonuclease site (CATG) in only the mutant allele.

from M.G. revealed the wild-type sequence. Thus M.G. was heterozygous for the $Arg^{201} \rightarrow$ His mutation (Fig. 2).

The $Arg^{201} \rightarrow$ His missense mutation introduces an *Nla* III restriction endonuclease site into the DNA. As shown in Fig. 3, exon 8 fragments amplified from peripheral leukocytes of control subjects are cut at a single *Nla* III site (lanes 6–8). By contrast, *Nla* III digestion of exon 8 fragments amplified from patient M.G.'s leukocytes produces two fragments (lanes 4 and 5). The smaller fragment is generated by cleavage at the *Nla* III site created by the G-to-A transition in the mutant allele. The autoradiographic intensity of the smaller fragment is less than that of the larger fragment, suggesting that the number of wild-type alleles is greater than the number of mutant alleles in DNA from M.G.'s leukocytes. These results confirm the presence of the mutation in the PCR fragments amplified from the patient's peripheral leukocytes, and they suggest that not all leukocytes contain the $Arg^{201} \rightarrow$ His mutation.

Analysis of exon 8 amplified from DNA of M.G.'s skin by DGGE or restriction fragment length polymorphism failed to demonstrate the presence of the $Arg^{201} \rightarrow$ His mutation. The

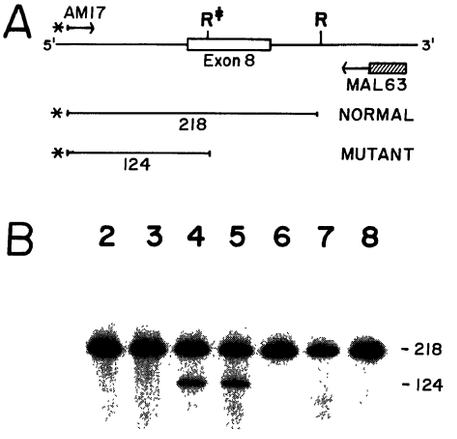

FIG. 3. Restriction enzyme analysis. (*A*) Two primers, AM-17 and MAL-63, were used to generate a PCR fragment encompassing exon 8. AM-17 was 5′ end labeled with ^{32}P (*). R shows the position of an *Nla* III restriction site in the PCR fragment. R‡ indicates the position of a second *Nla* III restriction site introduced by the mutation in the codon for Arg^{201}. The lines indicate the predicted sizes (in bp) of end-labeled fragments after digestion with *Nla* III for the normal and mutant alleles. (*B*) Polyacrylamide gel electrophoresis of the PCR fragments after digestion with *Nla* III. Note the presence of a second *Nla* III site in fragments amplified from the patient's leukocytes (lanes 4 and 5), confirming the presence of the mutation in the codon for Arg^{201}. Also note the relative intensities of the mutant and normal alleles in the patient's leukocytes (lanes 4 and 5) and the failure to detect the mutant allele in the patient's skin by this technique (lanes 2 and 3). Also shown are fragments amplified from peripheral leukocytes of an additional subject with MAS (lane 6) and two normal subjects (lanes 7 and 8).

exon 8 fragment amplified either from a *café-au-lait* lesion (lane 2) or from normal skin (lane 3) migrated as a single band on DGGE (Fig. 1) and did not produce an additional fragment after digestion with *Nla* III (Fig. 3). Exon 8 fragments amplified from cDNA synthesized from RNA prepared from the patient's skin also migrated as single bands on DGGE (not shown). The apparent absence of the $Arg^{201} \rightarrow$ His mutation in the patient's skin, coupled with the lower intensity of the band corresponding to the mutant allele on DGGE and restriction enzyme analysis, suggested that the patient is mosaic for the identified mutation. Because template competition by an excess of wild-type alleles could obviate our ability to detect low levels of the mutant allele, we employed more specific techniques in an effort to detect the $Arg^{201} \rightarrow$ His in the patient's skin. An allele-specific primer (WFS-2) was designed with a thymidine at the 3′ terminus to facilitate preferential annealing with the exon 8 fragment containing the G-to-A transition (22, 23). An additional internal mismatch was introduced near the 3′ end, to further destabilize annealing of WFS-2 with the wild-type allele. Under appropriate annealing conditions this primer preferentially amplified the mutant allele, such that a PCR product was amplified from DNA containing the mutant allele, while little or no product was obtained from DNA that contained only the normal allele. Moreover, this primer was able to amplify the mutant allele in the presence of a 1000-fold excess of the normal allele (data not shown). As shown in Fig. 4*A*, a PCR fragment was consistently amplified from DNA prepared from shave biopsy samples of both normal skin and the *café-au-lait* lesion. We consistently obtained more product from the affected skin. Similar results were obtained with a shorter allele-specific primer (WFS-5) that contained only one mismatch at the 3′ terminus (data not shown).

Allele-specific PCR was not 100% specific, as performing the PCR for more than 40 cycles or annealing at lower temperatures allowed for amplification of the normal allele. Therefore, we employed the SNuPE technique to confirm our results (24). This technique utilized a primer whose 3′ terminus is immediately adjacent to the identified mutation (WFS-6), a purified PCR fragment encompassing exon 8, and a single radiolabeled nucleotide ([^{32}P]dTTP) that would base pair with the G-to-A transition product. The primer was extended by a single nucleotide in a template-dependent reaction only in the presence of the mutant allele. SNuPE demonstrated the presence of the $Arg^{201} \rightarrow$ His mutation in the patient's normal skin, *café-au-lait* lesion, and leukocytes (Fig. 4*B*, lanes 2–5). However, no extension product was obtained with genomic DNA from normal subjects (lanes 7 and 8) or with cDNA synthesized from RNA isolated from M.G.'s skin (lanes 9 and 10).

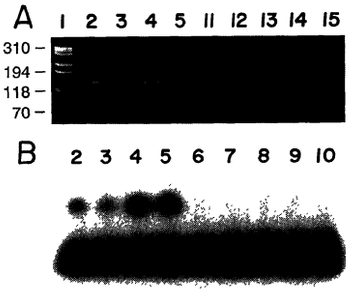

FIG. 4. Allele-specific PCR and SNuPE. (*A*) The primers AM-17 and WFS-2 were used to specifically amplify the mutant allele from genomic DNA (250 ng each in lanes 4–14, 500 ng in lanes 2 and 3). Under appropriate conditions no product was obtained from normal controls (lanes 11–14) or in the absence of genomic DNA (lane 15). However, product was amplified from two samples of the patient's leukocytes (lanes 4 and 5), from the patient's *café-au-lait* lesion (lane 2), and from the patient's normal skin (lane 3). We consistently obtained less product from the patient's normal skin than from his *café-au-lait* lesion. (*B*) The primer WFS-6 was extended by a single radioactive nucleotide ([α-^{32}P]dTTP) in the presence of a purified exon 8 fragment spanning the mutant allele. Note that incorporation occurred with PCR products prepared from the patient's leukocytes (lanes 4 and 5), affected skin (lane 2) and normal skin (lane 3), but not from another patient with MAS (lane 6) or two normal controls (lanes 7 and 8). No incorporation was seen with a PCR product spanning exon 8 amplified from cDNA prepared from RNA isolated from the patient's *café-au-lait* lesion (lane 9) or normal skin (lane 10).

DISCUSSION

The lack of documented inheritance of MAS, the segmental distribution of the bone and skin lesions, and the variability in metabolic abnormalities have suggested that MAS results from a postzygotic somatic cell mutation with mosaic distribution of the mutation-bearing cells (1). The similarity between the autonomously hyperfunctioning endocrine cells in MAS and sporadic endocrine tumors containing activating mutations within exon 8 or exon 9 of $G_s\alpha$ prompted us to analyze these regions in patients with MAS. We describe here the presence of a missense mutation, $Arg^{201} \rightarrow$ His, in one exon 8 of one allele of the gene encoding $G_s\alpha$ (*GNAS1*), from a patient with MAS; exon 9 sequences were normal. Whereas the exon 8 mutation was present in a large fraction of peripheral leukocytes, our results indicated that the defect was present in very few skin cells. These observations have two important implications: they suggest a molecular mechanism for MAS and support the previous suggestion that patients with MAS are mosaic.

While the idea has not been directly tested, several lines of evidence suggest that the $Arg^{201} \rightarrow$ His mutation is the basis for autonomous function and cellular proliferation of our patient's melanocytes, thyroid follicular epithelia, osteoblasts, and renal tubular cells. Previous studies have demonstrated the importance of the arginine at position 201 in the function of the $G_s\alpha$ protein. Arg^{201} of the $G_s\alpha$ chain is the site of ADP-ribosylation by cholera toxin. This post-translational modification inhibits the GTPase activity of $G_s\alpha$ and causes unregulated activation of adenylyl cyclase. More recently, abnormal forms of $G_s\alpha$ that fail to be ADP-ribosylated by cholera toxin have been identified in a subset of human GH-secreting pituitary tumors. Plasma membranes prepared from these tumors demonstrate markedly elevated adenylyl cyclase activity in the absence of GH-releasing hormone (25). Molecular analysis of these tumors revealed somatic mutations in $G_s\alpha$ that replaced Arg^{201} (CGT) with either Cys (TGT) or His (CAT) or replaced Gln^{227} (CAG) with Arg (CGG) (12). *In vitro* mutagenesis studies demonstrated that replacement of Arg^{201} reduces the GTPase activity of $G_s\alpha$ by approximately 30-fold and enhances the ability of $G_s\alpha$ to stimulate adenylyl cyclase (12, 26). Moreover, expression of the $Arg^{201} \rightarrow$ His $G_s\alpha$ protein in $G_s\alpha$-deficient S49 cyc$^-$ murine lymphoma cells reproduces the adenylyl cyclase phenotype observed in the GH-secreting tumors containing the mutant $G_s\alpha$. Finally, the cholera toxin gene has been placed under the control of the GH promoter in a transgenic mouse model. These mice displayed somatotroph proliferation, pituitary hyperplasia, elevated GH, and gigantism, indicating that alteration of Arg^{201} in $G_s\alpha$ is sufficient to activate adenylyl cyclase constitutively and cause autonomous GH secretion and cellular proliferation (27).

Clinical studies of patients with MAS and GH excess have demonstrated that they are indistinguishable biochemically from patients with GH-secreting pituitary adenomas. These observations have led to the speculation that GH excess in patients with MAS is caused by an intracellular defect in somatotroph differentiation or regulation (6). We suggest that the $Arg^{201} \rightarrow$ His mutation could represent such a defect; its presence in hormonally responsive cells of patients with

MAS could explain not only the GH excess but also the other endocrinopathies seen in MAS. In each case autonomous cellular activity could result from constitutive activation of adenylyl cyclase by the activating mutation of $G_s\alpha$.

Happle has proposed that the distribution of *café-au-lait* lesions in MAS is due to the growth of two different cell populations during early embryogenesis and reflects underlying mosaicism (1). The conclusion that the $G_s\alpha$ mutation we have identified is present in a mosaic distribution is supported by several lines of evidence. First, using conventional PCR techniques, we detected the $Arg^{201} \rightarrow$ His $G_s\alpha$ mutation in DNA from the patient's peripheral blood leukocytes but not in DNA from the patient's skin. This dramatic difference indicates that the mutation was not present in all cells. Second, semiquantitative analysis revealed that the mutant allele represented less than half of the alleles in this patient's leukocytes. Third, the exon 8 mutation was not detected in DNA from Epstein–Barr virus-transformed lymphoblasts (data not shown), suggesting that the mutation was not present in all blood cells (i.e., B lymphocytes). Finally, although standard techniques failed to reveal the $G_s\alpha$ mutation in DNA prepared from the patient's skin, allele-specific techniques indicated that the mutant allele was present in low numbers. We estimate that this mutation is present in less than 1 in 8 skin cells but in more than 1 in 1000 skin cells. This fits well with our estimate of the number of melanocytes in the shave biopsy sample being 1 in 50 to 1 in 100 cells. Our inability to detect the mutant allele in cDNA may be due to the very low number of copies of the mutant $G_s\alpha$ mRNA. The $Arg^{201} \rightarrow$ His mutation was present in DNA from normal skin and from a *café-au-lait* lesion, although we consistently saw evidence of higher levels of the mutant allele in affected skin.

Our findings support the results of Weinstein *et al.* (28). Using the techniques of PCR and allele-specific hybridization, these authors identified a mutation in Arg^{201}, either $Arg^{201} \rightarrow$ His or $Arg^{201} \rightarrow$ Cys, in pathologic specimens from each of the four patients with MAS. The mutant allele was present in highest concentration in affected endocrine tissues, but it was found in low levels in most tissues examined. These authors also identified the mutant allele in leukocytes but were unable to demonstrate the presence of the mutant allele in skin.

Taken together, these results suggest that MAS is a member of a growing family of diseases caused by somatic mosaicism of an autosomal dominant lethal mutation. On the basis of clinical evidence (2) and theoretical considerations (29) somatic mosaicism has been implicated as a cause of sporadic human diseases. Recent studies have identified the molecular mechanism of disease and demonstrated mosaicism in some examples. Hypomelanosis of Ito, a dermatologic disorder characterized by irregular hypopigmented patches on the trunk and limbs, has been associated with trisomy 18 mosaicism (30). Ornithine transcarbamoylase deficiency, a chromosome X-linked disorder that usually causes perinatal demise, has been associated with large deletion of the ornithine transcarbamoylase structural gene in a fraction of the cells in one mildly affected male patient (31). Somatic as well as germ-line mosaicism has been identified in the mothers of probands with hemophilia A, which involves a large deletion in the factor VIII gene (32), and hemophilia B, which involves a point mutation in the factor IX gene (33).

Finally, this study offers a note of caution to investigators who are studying genetic diseases by examining DNA prepared from peripheral blood leukocytes. We would have missed the mosaic nature of this disease had we not also examined DNA prepared from skin biopsy samples. In situations where inheritance of an abnormal phenotype is not well documented one must always consider the possibility that a somatic mutation rather than a germ-line mutation is the basis for the disorder.

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1. Happle, R. (1986) *Clin. Genet.* **29,** 321–324.
2. Happle, R. (1987) *J. Am. Acad. Derm.* **16,** 899–906.
3. Foster, C. M., Ross, J. L., Shawker, T., Pescovitz, O. H., Loriaux, D. L., Cutler, G. B. & Comite, F. (1984) *J. Clin. Endocrinol. Metab.* **58,** 1161–1165.
4. Feuillan, P. P., Shawker, T., Rose, S. R., Jones, J., Jeevanram, R. K. & Nisula, B. C. (1990) *J. Clin. Endocrinol. Metab.* **71,** 1596–1601.
5. Mauras, N. & Blizzard, R. M. (1986) *Acta Endocrinol. Suppl.* **279,** 207–217.
6. Cuttler, L., Jackson, J. A., uz-Zafar, M. S., Levitsky, L., Mellinger, R. C. & Frohman, L. A. (1989) *J. Clin. Endocrinol. Metab.* **68,** 1148–1154.
7. Jones, D. T. & Reed, R. R. (1987) *J. Biol. Chem.* **262,** 14241–14249.
8. Gilman, A. G. (1984) *Cell* **36,** 577–579.
9. Patten, J. L., Johns, D. R., Valle, D., Eil, C., Gruppuso, P. A., Steele, G., Smallwood, P. M. & Levine, M. A. (1990) *N. Engl. J. Med.* **322,** 1412–1419.
10. Weinstein, L. S., Gejman, P. V., Friedman, E., Kadowaki, T., Collins, R. M., Gershon, E. S. & Spiegel, A. M. (1990) *Proc. Natl. Acad. Sci. USA* **87,** 8287–8290.
11. Levine, M. A., Downs, R. W., Jr., Moses, A. M., Breslau, N. A., Marx, S. J., Lasker, R. D., Rizzoli, E. R., Aurbach, G. D. & Spiegel, A. M. (1983) *Am. J. Med.* **74,** 545–556.
12. Landis, C. A., Masters, S. B., Spada, A., Pace, A. M., Bourne, H. R. & Vallar, L. (1989) *Nature (London)* **340,** 692–696.
13. Lyons, J., Landis, C. A., Griffith, H., Vallar, L., Grunewald, K., Feichtinger, H., Duh, Q. Y., Clark, O. H., Kawasaki, E., Bourne, H. R. & McCormick, F. (1990) *Science* **249,** 655–659.
14. Suarez, H. G., du Villard, J. A., Caillou, B., Schlumberger, M., Parmentier, C. & Monier, R. (1991) *Oncogene* **6,** 677–679.
15. Marx, S. J., Spiegel, A. M., Brown, E. M., Windek, R., Gardner, D. G., Downs, R. W., Jr., Attie, M. & Aurbach, G. D. (1978) *J. Clin. Endocrinol. Metab.* **47,** 1190–1197.
16. Levine, M. A., Ahn, T. G., Klupt, S. F., Kaufman, K. D., Smallwood, P. M., Bourne, H. R., Sullivan, K. A. & Van Dop, C. (1988) *Proc. Natl. Acad. Sci. USA* **85,** 617–621.
17. Kawasaki, E. S. (1990) in *PCR Protocols: A Guide to Methods and Applications*, eds. Innis, M. A., Gelfand, D. H., Sninsky, J. J. & White, T. J. (Academic, San Diego), pp. 21–27.
18. Sheffield, V. C., Cox, D. R., Lerman, L. S. & Myers, R. M. (1989) *Proc. Natl. Acad. Sci. USA* **86,** 232–236.
19. Myers, R. M., Maniatis, T. & Lerman, L. S. (1987) *Methods Enzymol.* **155,** 501–527.
20. Maniatis, T., Fritsch, E. F. & Sambrook, J. (1982) *Molecular Cloning: A Laboratory Manual* (Cold Spring Harbor Lab., Cold Spring Harbor, NY).
21. Ausubel, F. M., Brent, R., Kingston, R. E., Moore, D. D., Seidman, J. G., Smith, J. A. & Struhl, K. (1989) *Current Protocols in Molecular Biology* (Wiley, New York).
22. Stork, P., Loda, M., Bosari, S., Wiley, B., Poppenhusen, K. & Wolfe, H. (1991) *Oncogene* **6,** 857–862.
23. Norby, S., Lestienne, P., Nelson, I. & Rosenberg, T. (1991) *Biochem. Biophys. Res. Commun.* **175,** 631–636.
24. Kuppuswamy, M. N., Hoffmann, J. W., Kasper, C. K., Spitzer, S. G., Groce, S. L. & Bajaj, S. P. (1991) *Proc. Natl. Acad. Sci. USA* **88,** 1143–1147.
25. Vallar, L., Spada, A. & Giannattasio, G. (1987) *Nature (London)* **330,** 566–568.
26. Freissmuth, M. & Gilman, A. G. (1989) *J. Biol. Chem.* **264,** 21907–21914.
27. Burton, F. H., Hasel, K. W., Bloom, F. E. & Sutcliffe, J. G. (1991) *Nature (London)* **350,** 74–76.
28. Weinstein, L. S., Shenker, A., Gejman, P. V., Merino, M. J., Friedman, E. & Spiegel, A. M. (1991) *N. Engl. J. Med.* **325,** 1688–1695.
29. Hall, J. G. (1988) *Am. J. Hum. Genet.* **43,** 355–363.
30. Chitayat, D., Friedman, J. M. & Johnston, M. M. (1990) *Am. J. Med. Genet.* **35,** 422–424.
31. Maddalena, A., Sosnoski, D. M., Berry, G. T. & Nussbaum, R. L. (1988) *N. Engl. J. Med.* **319,** 999–1003.
32. Higuchi, M., Kochhan, L. & Olek, K. (1988) *Mol. Biol. Med.* **5,** 23–27.
33. Taylor, S. A. M., Deugau, K. V. & Lillicrap, D. P. (1991) *Proc. Natl. Acad. Sci. USA* **88,** 39–42.